LINCOLN UNIVERSITY COLLEGE
DKU016 (B)

Faculty of Science

Laboratory Manual

Advance Microbiology

Bachelor of Biotechnology (Hons.)

Edited By: Dr. Farzana Yasmin

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LINCOLN UNIVERSITY COLLEGE
Wisma Lincoln, No. 12,14,16 & 18,
Jalan SS 6/12, Off Jalan Perbandaran
47301 Petaling, Jaya,
Selangor Darul Ehsan, Malaysia
Tel.: +603-7806 3478
Fax: +603-7806 3479
Toll Free: 1-300-880-111
E-mail: lucp@lincoln.edu.my
info@lincoln.edu.my
Web: www.lucp.net
www.lincoln.edu.my

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Advance Microbiology

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- No entry without permission:
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- At work in the laboratory:
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- Before leaving the laboratory:
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure-numbered steps that need to be carried out.
- For consideration -some questions to help you think carefully about the results you have obtained.
- Materials-a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. Making Accurate Observations

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. Presenting Quantitative Results

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

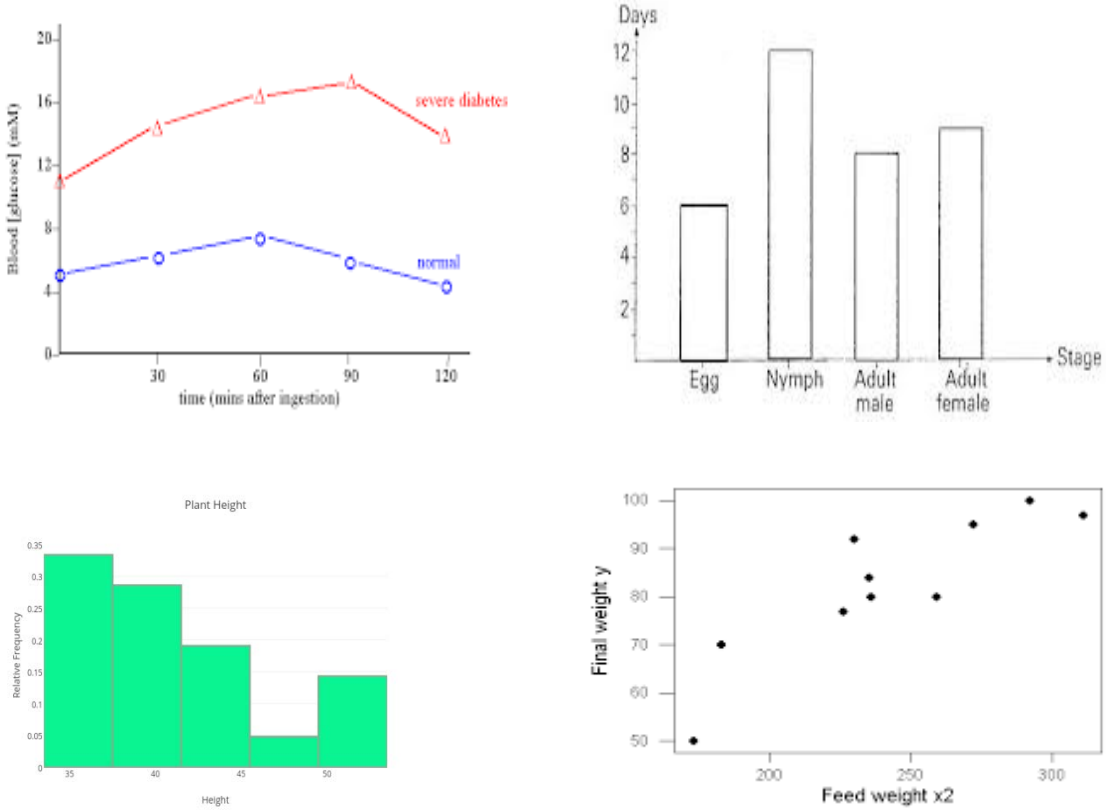


Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods

- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results

- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion

- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion

- Restate your conclusion.
- Restate important results.

Literature Cited

- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement

- State any appropriate acknowledgement that you think is necessary.

Practical 1

Title: Isolation of normal flora

Objectives:

After completing the practical, you will be able:

1. To isolate normal flora

Introduction:

The mouth and nose harbour many species of microorganisms. Some species of the respiratory tract are pathogenic. In healthy individuals, they are held at bay by immune defenses. However, when injury to the respiratory mucosa occurs, the pathogens are capable of multiplying and causing disease. In the mouth, there are a number of species that are essentially parasites. Their growth alters the conditions of the mouth such that the individual is more susceptible to tooth decay and ulcerations.

Some of the normal flora of the mouth and nose that are opportunistic pathogens include:
Streptococcus mitis: Causes ulcerations on the root of an injured or diseased tooth.
Streptococcus pyogenes: Causes strep throat.
Candida albicans: Yeast that causes "thrush" in small children, elderly people, or immunocompromised individuals.
Streptococcus pneumoniae: Can cause pneumonia following a respiratory infection, or if tissue resistance is low.

In this practical, you will look at some of the microflora that are present in your mouth and nose.

Specimen handling:

1. Throat swab
2. Skin swab
3. Mixed culture broth

Materials:

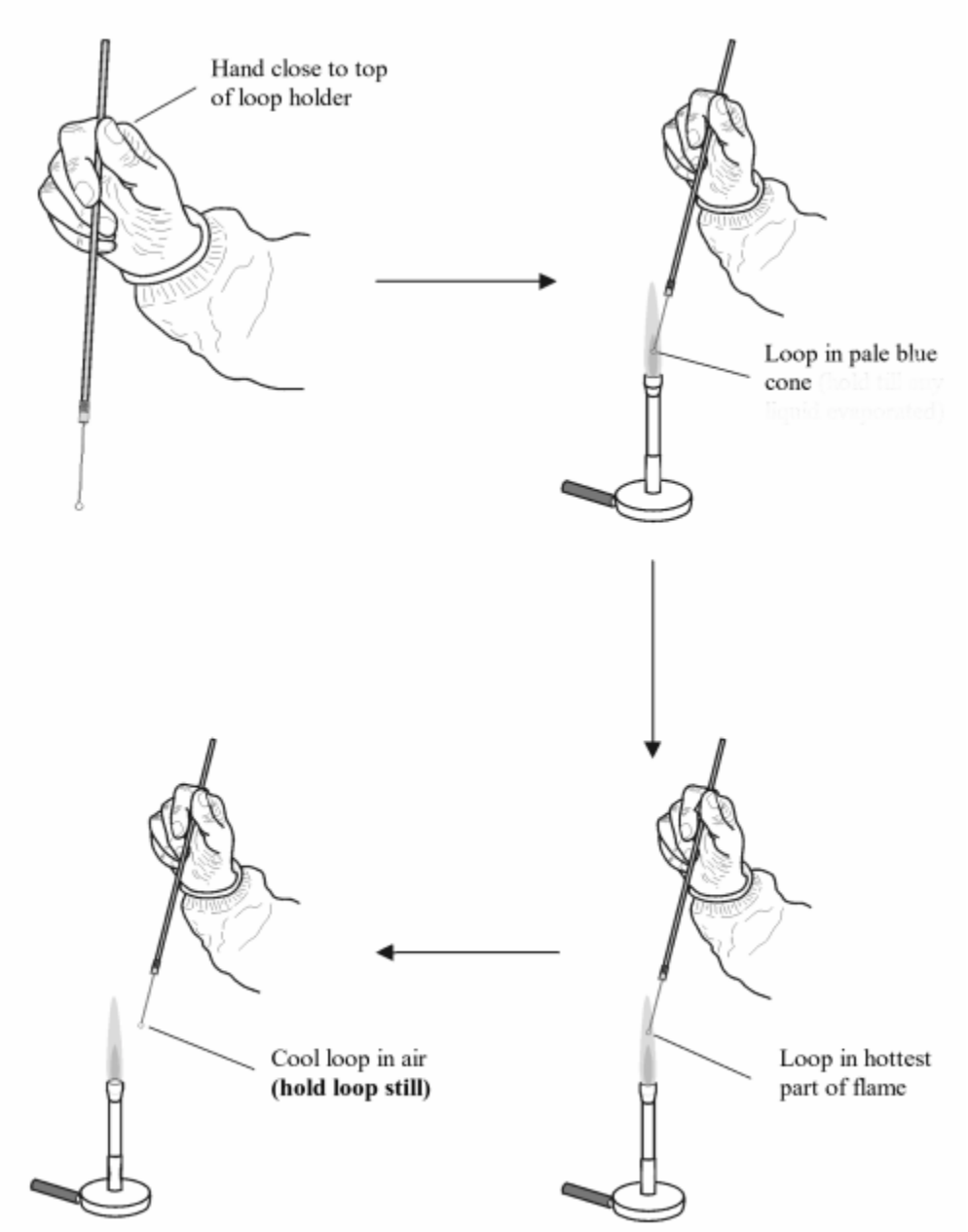
1. Nutrient Agar (NA)
2. McConkey Agar (MAC)
3. Eosin methylene blue (EMB)
4. Blood agar (BA)
5. Sterile cotton swabs
6. Tongue depressors
7. Sterile distilled water
8. Candle (CO_2) jar
9. Wire loop
10. Bunsen burner

Procedure:

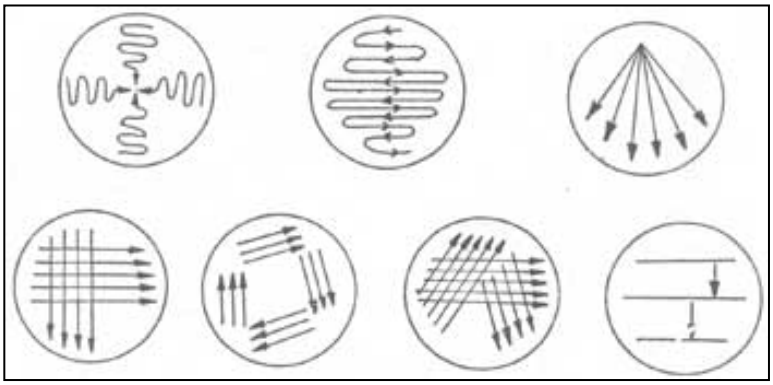
A. Streaking technique

1. Label the bottom of each set of agar plates provided (NA, MAC, and EMB) with group number, date, and the name of the specimens.
2. Take the samples as described below:
   a. Throat swab: Using a sterile tongue depressor, depress the tongue and gently rub the sterile swab across the throat area. Inoculate the sample in the quarter of the agar plates. Streak all plates following the standard streak plate technique.
   b. Skin swab: Moist a sterile swab by dipping in sterile normal saline and swab the skin surface of the forearm. Inoculate and streak the sample on the agar as in 2a.
   c. Mixed culture broth: With a sterile swab, streak the sample on the agar as in 2a.
3. Incubate the plates in the inverted position at 37°C for 24 hours and observe the plates for growth and colony morphology.

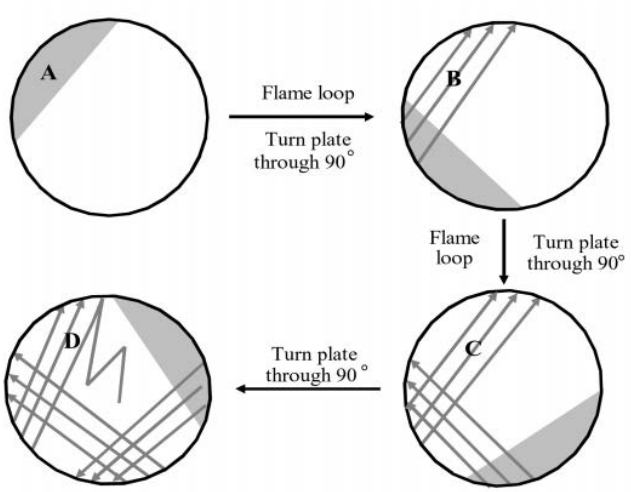
Flaming the loop



Different streaking techniques



Streak plate



Procedure:

1. Take one loop full of the sample using the sterilized loop.
2. Streak the sample over one quarter of the sterile Petri dish (Quadrant 1).
3. Sterilize loop in flame of Bunsen burner. Allow the loop to cool without waving it about.
4. Place the loop on the next quadrant of Petri dish, next to quadrant 1. Gently drag the loop into quadrant 1 a few times, to obtain just a bit of bacteria from that first sample, then spread that material over quadrant 2.
5. Again sterilize the loop in the flame of the Bunsen burner, and allow the loop to cool without waving it about.
6. Place loop in the next quadrant of Petri dish, adjacently to quadrant 2. Gently drag the loop into quadrant 2 a few times, to obtain just a bit of bacteria from that sample, then spread that material over quadrant 3.
7. Again sterilize the loop in the flame of the Bunsen burner, and allow the loop to cool without waving it about.

8. Place the loop in next quadrant of Petri dish, adjacent to quadrant 3. Gently drag the loop into quadrant 3 a few times, to obtain just a bit of bacteria from that sample, then spread that material over quadrant 4.

Results:

A. Colony morphology observation (Throat and skin swab)

Sample	Colony morphology and other characteristic features							
	Aerobic				Anaerobic			
	NA	MAC	EMB	BA	NA	MAC	EMB	BA
Throat								
Skin								

A. Colony morphology observation (Mixed broth)

Sample	Aerobic			
	NA	MAC	EMB	BA
Mixed culture broth				

Questions:

1. Explain the differences between the normal flora and pathogenic microbes.
2. How can the normal flora become pathogenic to human?
3. Define colony morphology and why it is important in bacterial identification?
4. Explain the importance of aseptic technique used in microbiology (taking the sample and culturing).
5. Describe five aseptic techniques that you practice in this investigation.

Practical 2
Title: Culture technique for fungi

Objective:

After completing the practical, you will be able:

1. To learn the technique of culture fungi

Introduction:

Fungi are eukaryotic organisms that can cause diseases to human. Fungal infections can be classified according to the tissues that are initially colonized. Infections of fungi include superficial mycoses, cutaneous mycoses and subcutaneous mycoses. The diagnosis of fungal infection requires observation of the fungal elements under the microscope.

Materials:

- Sabouraud agar
- Sterile Petri dish
- Filter paper (9 cm diameter)
- U-shaped glass rod
- Microscope slides and coverslips (Sterile)
- Sabouraud's plate with mixed culture of fungi
- Sterile Sabouraud's agar plate
- Lactophenol cotton blue stain
- Glass capillary tube
- Scalpel
- Inoculating needle
- Sterile distilled water
- 95% ethanol
- Forceps

Procedures:

A) Slide culture preparation

- Aseptically, with a pair of forceps, place a sheet of sterile filter paper in a Petri dish.
- Place a sterile U-shaped glass rod on the filter paper. (Rod can be sterilized by flaming, if held by forceps.)
- Pour enough sterile water (about 4 ml) on filter paper to completely moisten it.
- With forceps, place a sterile slide on the U-shaped rod
- Gently flame a scalpel to sterilize, and cut a 5 mm square block of the medium from the plate of Sabouraud's agar or Emmons' medium.
- Pick up the block of agar by inserting the scalpel and carefully transfer this block aseptically to the centre of the slide.
- Inoculate four sides of the agar square with spores or mycelial fragments of the fungus to be examined. Be sure to flame and cool the loop prior to picking up spores.
- Aseptically, place a sterile cover glass on the upper surface of the agar cube.
- Place the cover on the Petri dish and incubate at room temperature for 48 hours.
- After 48 hours, examine the slide under low power. If growth has occurred there will be growth of hyphae and production of spores. If growth is inadequate and spores are not evident, allow the mold to grow for another 24–48 hours before making the stained slides.

B) Application of stain

- Place a drop of lactophenol cotton blue stain on a clean microscope slide.
- Remove the cover glass from the slide culture and discard the block of agar.
- Add a drop of 95% ethanol to the hyphae on the cover glass. As soon as most of the alcohol has evaporated place the cover glass, mold side down, on the drop of lactophenol cotton blue stain on the slide. Examine the slide under microscope

Results:

Questions:

1. State the differences between hyphae and mycelium.
2. State the differences between the molds and yeasts.
3. Describe the advantages of slide culture technique.
4. Name any TWO culture media used to grow the fungi?

Practical 3
Title: Enumeration of microorganisms

Objective:
After completing the practical, you will be able:

1. To count the number of microorganisms using direct and plate count techniques

Introduction:

To determine rates of microbial growth and death, it is necessary to enumerate microorganisms, that is, to determine their numbers. It is also often essential to determine the number of microorganisms in a given sample. For example, the ability to determine the safety of many foods and drugs depends on knowing the levels of microorganisms in those products. A variety of methods has been developed for the enumeration of microbes. These methods measure cell numbers, cell mass, or cell constituents that are proportional to cell number.

1. Direct Count Using a Counting Chamber

In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed.. It is constructed in such a manner that the coverslip, slide, and ruled lines delimit a known volume. It is constructed in such a manner that the coverslip, slide, and ruled lines delimit a known volume. The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.

Procedure:

1. Pipette 1.0 ml of the sample of *E. coli* into a tube containing 1.0 ml of the dye methylene blue. This gives a 1/2 dilution of the sample.
2. Using a Pasteur pipette, fill the chamber of a Petroff-Hausser counting chamber with this 1/2 dilution.
3. Place a coverslip over the chamber and focus on the squares using 400X (40X objective).
4. Count the number of bacteria in 5 large double-lined squares. For those organisms on the lines, count those on the left and upper lines but not those on the right and lower lines. Divide this total number by 5 to find the average number of bacteria per large square.
5. Calculate the number of bacteria per cc as follows:

The number of bacteria per cc =

The average number of bacteria per large square X

The dilution factor of the large square (1,250,000) X

The dilution factor of any dilutions made prior to placing the sample in the counting chamber, such as mixing it with dye (2 in this case)

Results:

2. The plate count (viable count)

The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted and then plated out on an agar surface in such a manner that single isolated bacteria form visible isolated colonies , the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

For example, if a plate containing a 1/1,000,000 dilution of the original ml of sample shows 150 colonies, then 150 represents 1/1,000,000 the number of CFUs present in the original ml. Therefore the number of CFUs per ml in the original sample is found by multiplying 150 x 1,000,000 as shown in the formula below:

The number of CFUs per ml of sample =

The number of colonies (30-300 plate) X

The dilution factor of the plate counted

In the case of the example above 150 x 1,000,000 = 150,000,000 CFUs per ml.

For a more accurate count it is advisable to plate each dilution in duplicate or triplicate and then find an average count.

Procedure:

A. Plate Count

1. Take 6 dilution tubes, each containing 9.0 ml of sterile saline. Aseptically dilute 1.0 ml of a sample of *E. coli* .
a. Mix the tube thoroughly by either holding the tube in one hand or vigorously tapping the bottom with the other hand or by using a vortex mixer. This is to assure an even distribution of the bacteria throughout the liquid.
b. Using the same procedure, aseptically withdraw 1.0 ml from the first dilution tube and dispense into the second dilution tube. Continue doing this from tube to tube as shown until the dilution is completed. Your instructor will demonstrate these pipetting and mixing techniques.
2. Using a new 1.0 ml pipette, aseptically transfer 0.1 ml from each of the last three dilution tubes onto the surface of the corresponding plates of trypticase soy agar.
3. Using a turntable and sterile bent glass rod immediately spread the solution over the surface of the plates.
4. Incubate the inoculated plates for 24 to 48 hours at 37°C in incubator.

Results:

A. Plate Count

1. Choose a plate that appears to have between 30 and 300 colonies.
2. Count the exact number of colonies on that plate using the colony counter (as demonstrated by your instructor).
3. Calculate the number of CFUs per ml of original sample as follows:

The number of CFUs per ml of sample =

The number of colonies (30-300 plate) X

The dilution factor of the plate counted

4. Record your results.

Questions:

1. Explain the differences between the normal flora and pathogenic microbes.
2. How can the normal flora become pathogenic to human?
3. Define colony morphology and why it is important in bacterial identification?
4. Explain the importance of aseptic technique used in microbiology (taking the sample and culturing).
5. Describe five aseptic techniques that you practice in this investigation.

Practical 4
Title: Antibiotic sensitivity test / Antibiogram

Objective:
After completing the practical, you will be able:
1. To learn method of broth dilution and Kirby-Bauer Disk Diffusion

Introduction:
The results of antibiotic sensitivity tests are very important in the choice of antibiotic to be administered to the patient. In addition, it is also important in the case of bacterial resistance towards certain antimicrobial drugs. The terms Minimum Inhibitory Concentration (MIC) is used in order to report the lowest concentration of drugs that inhibits the growth of the organism. There are two methods to determine the MIC, namely Broth Dilution Method and Kirby-Bauer Disk Diffusion Method.

A. Broth dilution method

Materials:
1. Sterile test tubes with cap
2. Ampicillin with concentration of 100 μg/ml
3. Sterile 0.5 ml, 1 ml and 10 ml pipettes
4. Bulb or other aspiration device for pipette
5. Overnight plate culture of *Escherichia coli*
6. Bunsen burner
7. McFarland 0.5 standard
8. Normal saline (9.9 ml)
9. 7 ml and 100ml nutrient broth in universal bottle

Procedures:

1. Place seven sterile tubes in a rack and label them with number 1-7.
2. Add 9.0 ml of sterile broth to each test tube.
3. Add 1.0 ml of the ampicillin broth to the first tube. Discard the pipette.
4. Transfer 1.0 ml from this tube into the second tube. Discard the pipette. Mix the content by using vortex.
5. With a fresh pipette, mix the contents of the second tube and transfer 1.0 ml to the third tube.
6. Continue the dilution process until tube number 6. The seventh tube receives no antibiotic.
7. After the contents of the sixth tube are mixed, discard 1.0 ml of broth so that the final volume in all tubes is 9.0 ml.
8. From the plate culture of *E. coli* prepare a suspension of the organism in 7 ml of nutrient broth equivalent to a McFarland 0.5 standard.
9. Transfer 0.1 ml of the *E. coli* suspension into a tube containing 9.9 ml of saline. Discard the pipette.
10. Mix the contents of the tube well using vortex. Add 0.1 ml of this organism suspension to the antibiotic containing broth tubes 1 through 6 and to the growth control tube. Mix the contents in all the tubes by using vortex.
11. Place the tubes in the incubator for 24 hours.
12. After 24-hour incubation, observe the turbidity of the test tubes. The minimum inhibitory concentration (MIC) is then determined.

NOTE:

i. *Do not mix the tube for too long.*
ii. *All of the equipment must be sterile and the experiment must be conducted with proper aseptic technique.*

Results:

Broth dilution method:

Questions:

1. Why is it important to have the Control test tube?

2. Why do we need to use the fresh pipette each time when transferring the solution?

3. Explain why we cannot mix the test tube contents for too long by using the vortex.

B. Kirby-Bauer disk diffusion method

Materials:

1. *E. coli* colony on agar plate
2. Nutrient Agar (NA) plate
3. Test tube with broth
4. 0.5 McFarland turbidity standard
5. Inoculating wire
6. Sterile cotton swab
7. Antibiotic disks

Procedures:

1. Pick up 2 to 3 colonies of *E. coli* on the agar plate and emulsify them in the sterile saline. Match the turbidity to that of McFarland o.5 standard.
2. Dip the sterile cotton swab into the culture solution. Remove excess fluid by rotating the cotton swab at the side of the tube.
3. Inoculate the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum.
4. Allow the MHA agar plate at room temperature for 2 to 3 minutes.
5. By using sterile forceps, place the antibiotic disk on the MHA agar.
6. Incubate the plate at 37°C for 24 hours.
7. Observe and measure the zone of inhibition after 24 hours incubation.

Results:

Antibiotic disk	Zone of inhibition (mm)				Interpretation
	R1	R2	R3	Average	

R= Replica

Questions:

1. Explain briefly the principle of this experiment.

2. Define the terms ‘resistant’ and ‘susceptible’ to the antimicrobial agents.

KIRBY-BAUER DISK DIFFUSION METHOD

E. coli and other enteric Gram Negative Rods
(Zone Diameter, nearest whole mm)

	Resistant	Intermediate	Susceptible
Amikacin (30 µg)	≤14	15-16	≥17
Ampicillin(10 µg)	≤13	14-16	≥17
Cefazolin (30 µg)	≤14	15-17	≥18
Gentamicin (10 µg)	≤12	13-14	≥15
Tetracycline (30 µg)	≤14	15-18	≥19
Ticarcillin (75 µg)	≤14	15-19	≥20
Trimethoprim (5 µg)	≤10	11-15	≥16
Tobramycin (10 µg)	≤12	13-14	≥15

Staphylococcus species
(Zone Diameter, nearest whole mm)

	Resistant	Intermediate	Susceptible
Cefazolin (30 µg)	≤14	15-17	≥18
Clindamycin (2 µg)	≤14	15-20	≥21
Erythromycin (15 µg)	≤13	14-22	≥23
Gentamicin (10 µg)	≤12	13-14	≥15
Oxacillin (1 µg)	≤10	11-12	≥13
Penicillin G (10 µg)	≤28	--	≥29
Tobramycin (10 µg)	≤12	13-14	≥15
Vancomycin (30 µg)	--	--	≥15

LINCOLN UNIVERSITY COLLEGE

Wisma Lincoln, No. 12, 14, 16 & 18,
Jalan SS 6/12,47301 Petaling Jaya,
Selangor Darul Ehsan, Malaysia.
Tel.: +603-7806 3478 Fax: +603-7806 3479
Toll Free: 1-300-880-111
E-mail: lucp@lincoln.edu.my
Web.: www.lucp.net

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